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Award Number: DAMD17-98-1-8217

TITLE: Genetic Susceptibility to Estrogen-Induced Mammary Cancers

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REPORT DATE: November 2000

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

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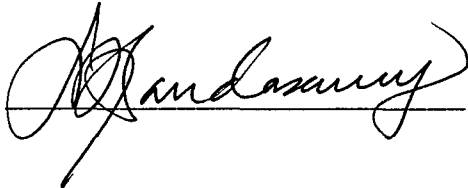
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1. AGENCY USE ONLY (Leave blank)		2. REPORT DATE November 2000	3. REPORT TYPE AND DATES COVERED Annual (1 Oct 99 - 1 Oct 00)	
4. TITLE AND SUBTITLE Genetic Susceptibility to Estrogen-Induced Mammary Cancers			5. FUNDING NUMBERS DAMD17-98-1-8217	
6. AUTHOR(S) James D. Shull, Ph.D.				
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) University of Nebraska Medical Center Omaha, Nebraska 68198-6810 E-MAIL: jshull@unmc.edu			8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012			10. SPONSORING / MONITORING AGENCY REPORT NUMBER	
11. SUPPLEMENTARY NOTES This report contains colored photos				
12a. DISTRIBUTION / AVAILABILITY STATEMENT DISTRIBUTION STATEMENT: Distribution authorized to U.S. Government agencies only (proprietary information, Nov 00). Other requests for this document shall be referred to U.S. Army Medical Research and Materiel Command, 504 Scott Street, Fort Detrick, Maryland 21702-5012.				12b. DISTRIBUTION CODE
13. ABSTRACT (Maximum 200 Words) Estrogens play a central role in the etiology of breast cancer. We have developed the ACI rats as a novel, physiologically relevant, and genetically defined model for the study of breast cancer etiology, treatment and prevention. In this model, estrogens rapidly induce mammary cancers that are estrogen dependent and exhibit aneuploidy. Susceptibility to estrogen-induced mammary cancers behaves as an incompletely dominant trait. Three loci have been identified to date that modify susceptibility in different genetic crosses. In this study we have evaluated allelic imbalances (AI) in a panel of mammary cancers induced by estrogen in female ACI/Copenhagen F1 progeny. AI is common and nonrandom in these cancers. Chromosome 1 exhibited the highest frequency of AI; 34% of examined marker/tumor pairs exhibited AI. Only 5 of 21 tumors did not exhibit AI at one or more marker on chromosome 1. In contrast, the frequency of AI on chromosome 9 was only 0.8%. AI was common on chromosomes 5, 18 and 2, which harbor the Emca1, Emca2 and Emca3 modifiers. These data suggest that non-random AI resulting from LOH and/or gene amplification may play a role in the etiology of estrogen induced mammary cancers.				
14. SUBJECT TERMS Breast Cancer			15. NUMBER OF PAGES 18	
			16. PRICE CODE	
17. SECURITY CLASSIFICATION OF REPORT Unclassified	18. SECURITY CLASSIFICATION OF THIS PAGE Unclassified	19. SECURITY CLASSIFICATION OF ABSTRACT Unclassified	20. LIMITATION OF ABSTRACT Unlimited	

Grant Number: DAMD17-98-1-8217
Project Title: Genetic Susceptibility to Estrogen-Induced Mammary Cancers
Principal Investigator: James D. Shull, Ph.D.
Date: October 25, 2000

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INTRODUCTION

Estrogens are inextricably implicated in the etiology of breast cancers in humans. Our laboratory has played a leading role in establishing the female ACI rat as a physiologically relevant animal model for the study of breast cancer etiology. We were the first to demonstrate that the naturally occurring estrogen, 17 β -estradiol (E2), induces rapid development of mammary cancers in the female ACI rat (1). In contrast, the genetically related Copenhagen (COP) rat strain is relatively resistant to E2-induced mammary cancers (2). Recent data from our laboratory indicate that the unique susceptibility of the ACI rat to E2-induced mammary cancers is inherited in reciprocal ACI x COP intercrosses as an incompletely dominant trait (3-5) that is conferred in part by a locus, *Emcal*, on rat chromosome 5 (3-5). In this USAMRMC funded research we are testing the hypothesis that genomic instability is an important contributing factor in the etiology of E2-induced mammary cancers. The primary experimental approach is to utilize a battery of polymorphic genetic loci to assess loss of heterozygosity (LOH) in mammary tumors induced by E2 in female F1 progeny generated by mating ACI and COP rats. We have demonstrated that E2-induced mammary cancers frequently exhibit genomic instability. Approximately two-thirds of the tumors examined exhibit aneuploidy (6), and all of the tumors examined exhibit allelic imbalances on one or more chromosomes.

BODY

This progress report summarizes work completed between May 15, 1999 and October 15, 2000. Initiation of this research was delayed as the Specific Aims and Statement of Work were revised to eliminate perceived overlap with an award from the National Cancer Institute. Progress relating to each of the revised Statement of Work Tasks is summarized herein.

Task 1. Each mammary tumor will be evaluated histologically to define its type as comedo or papillary carcinoma and to assess its extent of invasiveness. Year 1 and beyond as additional tumors become available.

This task has been accomplished for tumors obtained to date. All E2-induced mammary tumors were adenocarcinoma of the comedo, cribriform or papillary types, with the comedo form being the predominant cancer type. Invasive features were observed in a significant fraction of these mammary tumors.

Task 2. Cells will be isolated from each mammary tumor and analyzed by flow cytometry to quantify cellular DNA content and assess ploidy. DNA content different from 2N and 4N will be considered to be an indicator of aneuploidy. Year 1 and beyond as additional tumors become available.

Methods: Frozen mammary tumors and spleens from the tumor bearing animals were thawed on ice, cut into small pieces using a razor blade and mechanically dissociated in PBS using a Stomacher laboratory blender (Tekmar, Cincinnati, OH). The dissociated cells were pelleted by centrifugation, resuspended in RPMI culture media (Life Technologies, Gaithersburg, MD), filtered through Spectra/Mesh polymer macrofiltration filters (Spectrum, Laguna Hills, CA) and counted. Following dilution to approximately 1 million cells per ml in Vendelov's reagent the cells were analyzed using a FACStar Plus flow cytometer (Becton Dickinson, San Jose, CA). Cells prepared from normal spleen were used as an internal ploidy standard. The resulting data were analyzed using ModFit DNA analysis software (Becton Dickinson).

Results and Discussion: Flow cytometric analysis of DNA content within cells isolated from mammary cancers induced in female ACI rats by E2 indicated that the majority of these tumors exhibit genomic instability. Within a group of fifteen E2-induced mammary cancers, five (33%) of the tumors exhibited a normal DNA profile, where the majority of cells displayed a diploid DNA content. Seven (47%) tumors contained a significant fraction of cells which exhibited a hyperdiploid DNA content, two (13%) tumors contained a large fraction of cells that were tetraploid in their DNA content, and one (7%) of the 15 mammary tumors contained a large fraction of cells that were hypertetraploid in their DNA content. These data clearly illustrate that different E2-induced mammary carcinomas are heterogeneous with respect to ploidy, with approximately 66% of the examined tumors exhibiting clear features of aneuploidy. This observation is noteworthy as the mammary tumors that develop in rats and mice treated with dimethylbenz[a]anthracene have been reported to exhibit a diploid DNA profile. These data were included in a recent manuscript from our laboratory published in the Proceedings of the

National Academy of Sciences (97:2779-2784). Support from the USAMRMC was acknowledged in that publication. A reprint of the manuscript is appended to this progress report.

Task 3. A panel of appropriately located simple sequence repeat (SSR) loci will be identified that are polymorphic between the ACI and COP and the ACI and BN strains from among those mapped in the rat genome.

Results and Discussion: This task has been accomplished on a chromosome-by-chromosome basis and will be continued throughout the remainder of the research project. Analyses of tumors in ACI/COP F1 females are problematic for certain regions of the genome due to a low density of SSR markers that are polymorphic between these two rat strains. The density of polymorphic markers is much greater for the intended analysis of ACI/BN F1 females.

Task 4. DNA will be isolated from each mammary tumor as well as from the spleen of each mammary tumor bearing F1 animal. Each of the polymorphic SSR loci identified in #3 will be amplified using polymerase chain reaction (PCR) and commercially available primers. The amplified DNAs will be subjected to electrophoresis on denaturing acrylamide gels and the amount of DNA product generated from the ACI and COP or ACI and BN alleles at each locus will be quantified using a phosphorimager. LOH will be revealed as a strain specific loss of an SSR locus in the mammary tumor relative to the spleen from the same animal. Suspected LOH will be confirmed by repeating the analyses at flanking loci. Years 1 through 3 as warranted by new releases of the rat genome map.

and

Task 5. The resulting data will be analyzed to determine: 1) the extent of LOH occurring across the rat genome; 2) whether the observed LOH occurs selectively in specific regions of the genome; and 3) whether LOH within a specific region of the genome correlates with tumor type or invasiveness. Years 2 and 3.

Methods: The rat tissues used in this project are from experiments supported by NIH grant CA77876. In the NIH funded experiments, mammary tumors were induced in ACI/COP F1 and ACI/BN F1 female rats by treatment with E2, administered from subcutaneous Silastic implants as described by us previously (1). No funds from the USAMRMC were used for purchase or housing of live animals.

Methods for evaluating LOH were established and optimized. Genomic DNA was isolated from 10 mg of rat spleen and 25 mg of rat mammary tumor using QIAamp tissue kits (QIAGEN). Each DNA (30 ng) was amplified by PCR using Platinum Taq DNA Polymerase (Gibco) and standard methods as described in our original application; [32P]dCTP (Amersham) was included in each PCR reaction, resulting in generation of labeled PCR products. Oligodeoxynucleotide primers for amplifying the different polymorphic SSR loci on each of the rat chromosomes were obtained from Research Genetics, Inc. The PCR products were separated on 8% denaturing

polyacrylamide sequencing gels. Each PCR reaction was assayed in triplicate. Following drying, the gels were visualized by autoradiography on a Phosphorimager (Molecular Dynamics), and the intensities of the DNA bands generated from the ACI and COP alleles were quantified using ImageQuant software (Molecular Dynamics). The average (\pm standard deviation, $n = 3$) pixel intensity generated from the ACI and COP alleles from spleen and mammary tumor DNA was determined for each of several SSR loci. An LOH index for each polymorphic locus was calculated by: 1) dividing the intensity of the ACI product by the intensity of the COP product generated from each mammary tumor and spleen DNA; 2) comparing the ACI/COP ratio from the mammary tumor with the ACI/COP ratio from the spleen using Student's *t* test; and, 3) subsequently dividing the ACI/COP ratio from each mammary tumor by the ACI/COP ratio from each spleen. The standard deviation of each ACI/COP ratio ($n = 3$) rarely exceeded 10% of the mean. An LOH index that was equal to or greater than 1.25 was interpreted to suggest loss of the COP allele within the genome of the mammary tumor at the locus assayed. An LOH index that was equal to or less than 0.75 was interpreted to suggest loss of the ACI allele within the genome of the mammary tumor at the locus assayed.

Results and Discussion: Analyses of allelic imbalance (AI), resulting from deletion (LOH) or gene amplification, in mammary tumors induced by E2 in female ACI/COP F1 progeny is virtually completed on all rat chromosomes except chromosomes 6, 11, 16 and 19. Data accumulated to date indicate that AI in these tumors is common and nonrandom. The highest frequency of AI was observed on chromosomes 1, 5, 17, 18 and 20, whereas the lowest frequency of AI was observed on chromosomes 9, 12, 13, 14. An abstract describing these findings has been submitted for presentation at the 2001 meeting of the American Association for Cancer Research. A copy of this abstract is appended to this progress report. We anticipate that a manuscript describing these findings will be submitted early in 2001. Representative data are summarized here.

AI was evaluated at 5 polymorphic SSR markers on rat chromosome 1 in 21 tumors induced in ACI/COP females by E2. AI was observed at each of the five markers in a minimum of 3 tumors and a maximum of 9 tumors. Overall, AI was observed at 34% of the marker/tumor pairs for which informative data were generated. Only 5 of the 21 tumors did not exhibit AI at one or more markers. Of the 33 AIs observed, 25 (82%) resulted from loss of ACI alleles (or gain of COP alleles).

We have mapped to rat chromosome 2 a locus, *Emca3*, that modifies susceptibility to E2-induced mammary cancers in an ACI x BN intercross (unpublished data). We have evaluated AI at 6 polymorphic loci on chromosome 2. AI was observed at 14% of the informative marker/tumor pairs. Each of the markers exhibited AI in at least 1 and as many of 4 tumors. Eleven of the 21 tumors did not exhibit AI at any marker. AIs resulting from loss of ACI alleles were observed at the same frequency as AIs resulting from loss of COP alleles.

Emca1, a second modifier of susceptibility to E2-induced mammary cancers has been mapped by us to rat chromosome 5 (unpublished data). For each of 17 polymorphic markers evaluated on rat chromosome 5, AI was observed in at least 1 and as many as 7 of the 21 tumors. On average, an individual tumor exhibited AI at 20% (range 0-82%) of the markers. Approximately 65% of the observed AIs resulted from loss of COP alleles (or gain of ACI alleles). Only 1 tumor did

not exhibit any AI, 2 tumors exhibited AI at approximately 50% of the markers and 2 tumors exhibited AI at a majority of the chromosome 5 markers. Within the 4 tumors exhibiting AI at 50% or more of the markers, 3 tumors exhibited consistent loss of COP alleles and 1 exhibited consistent loss of ACI alleles.

AI was virtually lacking on rat chromosome 9. Although evaluated at 6 polymorphic markers, AI was observed at only one marker in a single tumor, a frequency of 0.8% for the informative marker/tumor pairs.

AI was evaluated at six polymorphic markers on rat chromosome 18, onto which the *Emca2* modifier of susceptibility to E2-induced mammary cancers has been mapped by us. AI was observed at each of these markers in a minimum of 3 and a maximum of 11 of the 21 mammary tumors. A total of 36 AI events were observed within the 133 informative marker/tumor pairs evaluated, an overall frequency of 29%. Of the 36 observed events, 22% resulted from loss of ACI alleles, whereas 78% resulted from loss of COP alleles.

Task 6. The data will be communicated to the scientific community through presentation at appropriate meetings and publication in peer reviewed journals. Years 2 through 3.

The data indicating that mammary tumors induced in ACI rats by E2 exhibit aneuploidy were communicated by Dr. Shull in an invited presentation at the Gordon Conference on Hormonal Carcinogenesis held in August of 1999 and were included in a recent manuscript published in the Proceedings of the National Academy of Sciences 97:2779-2784, 2000. Support from the USAMRMC was acknowledged in that publication. A reprint of the manuscript is appended to this progress report.

An abstract describing the genome wide screen of AI has been submitted for presentation at the 2001 meeting of the American Association for Cancer Research. A copy of this abstract is appended to this progress report. We anticipate that a manuscript describing these findings will be submitted early in 2001.

Task 7. All required progress reports and communications will be prepared and submitted to the Army Breast Cancer Research Program. Years 1 through 3.

In progress and to be continued as necessary.

KEY RESEARCH ACCOMPLISHMENTS

1. Flow cytometric analyses of E2-induced mammary tumors have been performed indicating that a large fraction of these tumors exhibit aneuploidy.
2. Methods for evaluating LOH have been established and optimized.
3. Appropriate polymorphic loci have been identified for evaluating LOH in mammary tumors induced in ACI/COP F1 rats by chronic treatment with E2.
4. AI has been evaluated on 16 of the 20 rat autosomes, revealing that AI is common and nonrandom in E2-induced mammary cancers.

REPORTABLE OUTCOMES

Manuscripts, abstracts, presentations: One manuscript has been published. Several oral presentations have included references to the data generated with support of this award. A minimum of one additional manuscript will be submitted in early 2000.

Patents: None.

Degrees: None.

Development of cell lines: None.

Informatics such as databases and animal models: None.

Funding applied for: None at present. Additional applications anticipated.

Employment or research opportunities: None.

CONCLUSIONS

Data generated to date suggest that genomic instability and AI are common events during E2-induced mammary tumorigenesis. It is anticipated that this information will enhance our understanding of the molecular events leading to mammary cancer development. Our research on estrogen-induced mammary tumorigenesis is illustrating for the first time the physiological relevance of the ACI rat as an animal model for the study of breast cancer etiology.

No changes in the research plan are anticipated at this time.

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Rat strain-specific actions of 17 β -estradiol in the mammary gland: Correlation between estrogen-induced lobuloalveolar hyperplasia and susceptibility to estrogen-induced mammary cancers

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Communicated by Jack Gorski, University of Wisconsin, Madison, WI, December 23, 1999 (received for review December 1, 1999)

The genetically related ACI and Copenhagen (COP) rat strains display diametrically opposed susceptibilities to mammary cancer development when treated chronically with 17 β -estradiol (E2). Here, we compare the actions of E2 on cell proliferation and lobuloalveolar development in the mammary glands of female ACI and COP rats. After 12 wk of E2 treatment, the mammary glands of ACI rats exhibited a significantly greater proliferative response to E2, compared with COP rats, as evidenced by quantification of S phase fraction and development of lobuloalveolar hyperplasia. Focal regions of atypical epithelial hyperplasia were observed in ACI, but not COP, rats. These strain differences were not because of differences in circulating E2, progesterone or, prolactin. Two-thirds of the induced mammary cancers in ACI rats exhibited aneuploidy. The E2-induced mammary cancers regressed when hormone treatment was discontinued, indicating that they were estrogen-dependent. Progesterone receptor was expressed by the great majority of epithelial cells within the E2-induced atypical hyperplastic foci and the mammary carcinomas, suggesting a link between these lesions. These data demonstrate a correlation between E2 action in the induction of mammary cell proliferation and atypical epithelial hyperplasia and genetically conferred susceptibility to E2-induced mammary cancers.

Epidemiologic studies suggest that estrogens are critical factors in breast cancer etiology (1–3). Supporting this link are the observations that early menarche, late menopause, late first full-term pregnancy, and nulliparity are each associated with increased risk of the disease, whereas oophorectomy prior to menopause significantly decreases breast cancer risk. A recent clinical trial demonstrated that the antiestrogen tamoxifen significantly reduces the incidence of breast cancer in women at high risk of developing the disease (4). One theory consistent with these data is that breast cancer risk is proportional to the cumulative number of ovulatory cycles and the repetitive stimulation of mammary cell proliferation by ovarian estrogens. At present, the molecular mechanisms through which estrogens contribute to the development of breast cancers are unknown.

The ACI rat appears unique among rat strains in that it is highly susceptible to estrogen-induced mammary cancers but rarely develops mammary cancers spontaneously (5–15). We have demonstrated that chronic treatment with 17 β -estradiol (E2) induces mammary cancers in virtually 100% of ovary-intact ACI rats with a median latency of approximately 20 wk (15). In contrast, the Copenhagen (COP) rat is resistant to the development of estrogen-induced mammary cancers (5–8, 16). This difference between the ACI and COP rat strains is particularly striking because these strains are closely related genetically; the ACI strain was derived from a cross between the August and COP strains (17). Because the ACI and COP rat strains exhibit diametrically opposed susceptibilities to E2-induced mammary

cancers (15, 16), they provide valuable models for studying the molecular mechanisms through which estrogens contribute to mammary cancer development.

In the present study, we have compared the ability of E2 to stimulate mammary cell proliferation and lobuloalveolar development in the mammary glands of female ACI and COP rats. Data presented herein reveal quantitative and qualitative differences in the manner in which the mammary epithelia of ACI and COP rats respond to E2, suggesting that genetic control of estrogen action in the mammary epithelium may contribute to the differing susceptibilities of these rat strains to E2-induced mammary cancers.

Materials and Methods

Care and Treatment of Animals. All procedures involving live animals were approved by the Institutional Animal Care and Use Committee of the University of Nebraska Medical Center. Female ACI and COP rats were obtained from Harlan–Sprague–Dawley and National Cancer Institute Breeding Program (Frederick, MD), respectively. The animals were housed in an accredited barrier facility under controlled temperature, humidity, and lighting conditions, and were fed a diet that was formulated in accordance with guidelines established by the American Society of Nutritional Science (18) and prepared as described previously (19, 20). Treatment with E2 was initiated at approximately 9 wk of age. Silastic tubing implants, empty or containing 27.5 mg of E2, were surgically inserted into the interscapular region as described previously (15, 16). Thereafter, each rat was examined twice weekly for palpable mammary cancers. Four hours preceding killing, each rat received an i.p. injection of BrdUrd (Sigma; 50 mg/kg body weight) solubilized in sterile PBS.

Collection, Processing, and Immunohistochemical Evaluation of Mammary Tissues. Following treatment with E2 for the indicated period of time, each animal was killed by decapitation, trunk blood was collected, and serum was stored at –80°C. The anterior pituitary gland was removed and weighed. Serum E2 (15), prolactin (PRL) (15), and progesterone (21) were measured by radioimmunoassay as described previously. The location and size of each mammary tumor were recorded at nec-

Abbreviations: COP, Copenhagen; DES, diethylstilbestrol; E2, 17 β -estradiol; PR, progesterone receptor; PRL, prolactin.

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Article published online before print: *Proc. Natl. Acad. Sci. USA*, 10.1073/pnas.050569097. Article and publication date are at www.pnas.org/cgi/doi/10.1073/pnas.050569097

ropsy. Mammary tissues, both grossly normal and tumors, were collected, fixed in Lillie's solution for 24 hr, processed, and embedded in paraffin. Portions of randomly selected mammary tumors were frozen in liquid nitrogen and stored at -80°C ; spleens from these animals were also frozen and stored at -80°C . Sections from each mammary tissue specimen were stained with hematoxylin and eosin for histologic characterization. The density of the mammary parenchyma within each gland was estimated by computer-assisted (Optimas, Seattle, WA) quantification of the percentage of total cross-sectional area comprised of mammary parenchyma. Mammary glands from each animal were also prepared as whole mounts stained with alum carmine (Sigma) (22). Mammary cells incorporating BrdUrd during the 4-hr period preceding killing were quantified immunohistochemically as described previously (16, 19). A minimum of 2,000 mammary cells per animal were scored for incorporation of BrdUrd. To identify cells expressing progesterone receptor (PR), mammary sections were deparaffinized, rehydrated in water, and heated in citrate buffer (pH 6.0) for 20 min. Tissues were then incubated as follows: (i) 20 min at room temperature in 3% hydrogen peroxide; (ii) 20 min at room temperature in goat serum diluted 1:100 in PBS; (iii) 60 min at 37°C in rabbit anti-PR serum (C-19; Santa Cruz Biotechnology) diluted 1:100 in PBS. Bound primary antibody was detected by using biotinylated goat anti-rabbit IgG followed by incubation with avidin-biotin-peroxidase complex (Vector Laboratories) and visualized by using 3,3'-diaminobenzidine tetrahydrochloride (Dako). Sections were counterstained with Meyer's hematoxylin.

Analysis of Cellular DNA Content by Flow Cytometry. Frozen mammary tumors and spleens from tumor-bearing animals were thawed on ice, minced, and mechanically dissociated in PBS using a Stomacher laboratory blender (Tekmar, Cincinnati). The dissociated cells were pelleted by centrifugation, resuspended in RPMI culture media (Life Technologies, Gaithersburg, MD), filtered through Spectra/Mesh polymer microfiltration filters (Spectrum, Laguna Hills, CA), and counted. Following dilution to approximately 1 million cells per ml in Vendelov's reagent (23), the cells were analyzed by using a FACScalibur flow cytometer (Becton Dickinson). Cells prepared from normal spleen were used in parallel samples as an internal ploidy standard. The resulting data were analyzed by using MODFIT DNA analysis software (Becton Dickinson).

Statistical Analysis of Data. Data are presented as the mean \pm SEM. Differences between means were assessed by using two-way ANOVA and Newman-Keul's post hoc test. P values < 0.05 were considered significant.

Results

Responsiveness of Mammary Epithelium to E2 Is Rat Strain-Specific.

The mammary epithelium of the female ACI rat exhibited a more robust proliferative response to administered E2 than did that of the female COP rat. After 12 wk of treatment with E2 (a time point that precedes appearance of palpable mammary cancers), cell proliferation in the mammary glands of female ACI rats was increased approximately 8-fold relative to that observed in untreated controls (Fig. 1A). Whereas approximately 0.5% of mammary cells of control ACI rats stained positive for BrdUrd, 3.9% of mammary cells of E2-treated ACI rats were BrdUrd-positive ($P < 0.01$). By contrast, E2 treatment increased cell proliferation within the mammary glands of female COP rats 3.5-fold ($P < 0.01$). Approximately 0.8% and 3.0% of mammary cells of untreated and E2-treated COP rats, respectively, were BrdUrd-positive (Fig. 1A). The 30% difference in the S phase fraction between the E2-treated ACI and COP rats was significant ($P < 0.01$).

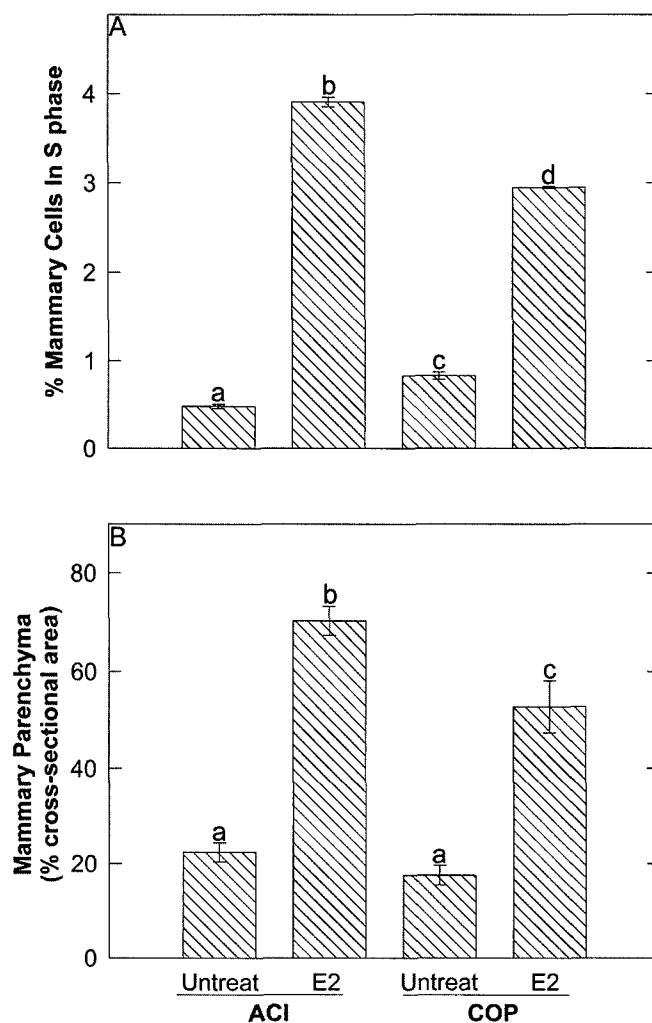


Fig. 1. The mammary epithelia of ACI and COP rats exhibited differing proliferative responses to E2. Ovary-intact ACI and COP rats were treated with E2 for 12 wk. (A) Mammary cells in S phase were identified by BrdUrd immunohistochemistry. Each data bar represents the mean \pm SEM ($n = 4-8$) number of mammary cells incorporating BrdUrd, expressed as a percent of total mammary cells. Differing lower case letters above data bars indicate statistical significance ($p \leq 0.05$) between group means as assessed by ANOVA. (B) The percentage of cross-sectional area comprised by mammary parenchyma was measured by computer-assisted image analysis. Each data bar represents the mean \pm SEM ($n = 7-8$).

The difference in the proliferative response of the ACI and COP mammary epithelia to E2 was reflected histologically (Fig. 1B, and see Fig. 2). The mammary glands of untreated virgin ACI (Fig. 2A and C) and COP (Fig. 2E and G) females were similar in appearance and consisted of branched ducts extending throughout the mammary fat pad and terminating in alveolar buds. Treatment with E2 for 12 wk induced ductal branching and extensive lobuloalveolar hyperplasia in both strains. However, the induction of lobuloalveolar hyperplasia was clearly more pronounced in the ACI rats (Fig. 2B and D) than in the COP rats (Fig. 2F and H). Quantitative analysis of stained sections indicated that the mammary parenchyma comprised 70% of cross-sectional area in E2-treated ACI rats compared with 53% in the treated COP rats (Fig. 1B). This difference, reflecting the greater density of lobules in the ACI glands, was statistically significant ($P < 0.01$) and was also evident upon examination of mammary gland whole mounts (Fig. 2D and H). Secretory activity was apparent in the mammary glands of treated ACI and

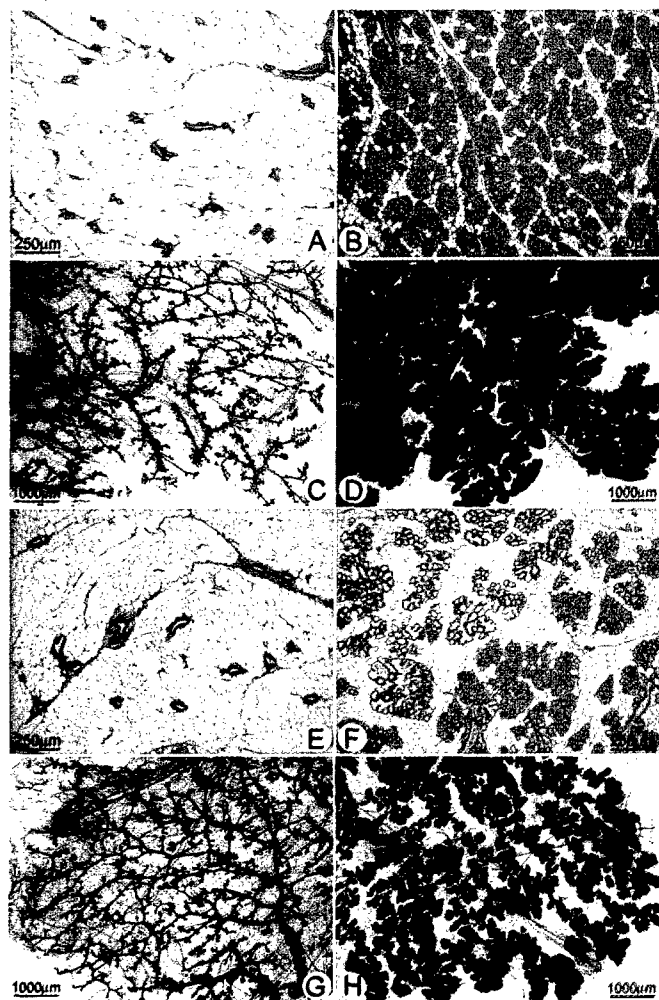


Fig. 2. The differing proliferative responses of the ACI and COP mammary glands were reflected in mammary histology. (A and E) Thin sections from untreated ACI and COP rats, respectively. (C and G) Whole mounts from these strains. The mammary glands of untreated ACI and COP rats appeared similar and were comprised of branched ducts terminating in alveolar buds. (B and F) Thin sections from E2-treated ACI and COP rats, respectively. (D and H) Whole mounts. Although E2 treatment induced ductal branching and lobuloalveolar hyperplasia in both ACI and COP rats, the response in ACI rats was more pronounced than in COP rats.

COP rats and, although variable, appeared more pronounced in the COP strain compared with the ACI strain (Fig. 2 B and F).

Strain Differences in Lobuloalveolar Hyperplasia Are Independent of Circulating E2, Progesterone, and Pituitary Tumor-Associated Hyperprolactinemia. The levels of E2, progesterone, and PRL in trunk blood serum were measured to ascertain their relationships to the observed strain difference in E2-induced lobuloalveolar hyperplasia. Serum E2 levels in treated animals did not differ between the two strains, averaging 165 ± 24 pg/ml and 306 ± 119 pg/ml (mean \pm SEM) in ACI and COP rats, respectively (data not shown). Progesterone levels were within the physiologic range and did not differ significantly as a consequence of E2 treatment or rat strain (data not shown). Treatment with E2 for 12 wk induced pituitary growth and hyperprolactinemia in both female ACI and COP rats. Pituitary weight was increased 3.4-fold ($P < 0.01$), from 12.0 ± 0.4 to 40.9 ± 2.9 mg, in female ACI rats, and 2.4-fold ($P < 0.01$), from 14.2 ± 0.4 to 33.5 ± 3.6 mg, in female COP rats (Fig. 3A). Similarly, treatment with E2 increased serum PRL from 4 ± 1 to 640 ± 114 ng/ml ($P < 0.01$)

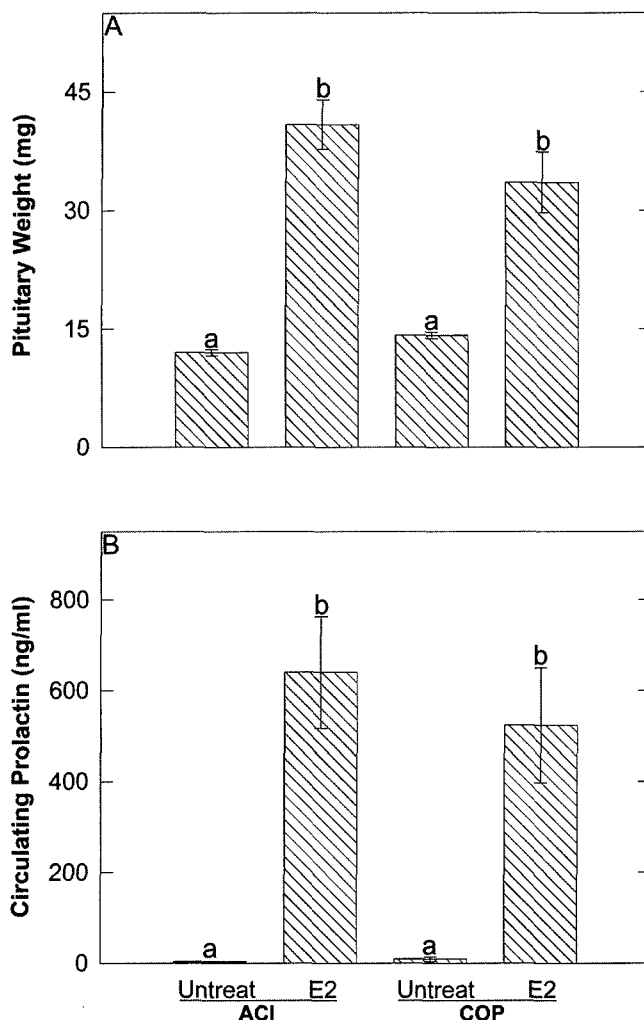


Fig. 3. E2 induced pituitary growth and hyperprolactinemia similarly in ACI and COP rats. (A) Each data bar represents the mean \pm SEM ($n = 7-8$) pituitary wet weight, which corresponds to pituitary cell number (20). Differing lower case letters above data bars indicate statistical significance ($p \leq 0.05$) between group means as assessed by ANOVA. (B) Each data bar represents the mean \pm SEM ($n = 7-8$) level of circulating PRL.

in female ACI rats, and from 9 ± 5 to 524 ± 118 ng/ml ($P < 0.01$) in female COP rats (Fig. 3B). The differences in pituitary weight (Fig. 3A) and serum PRL (Fig. 3B) between E2-treated ACI and COP rats were not significant. Therefore, the observed strain differences in E2-induced lobuloalveolar hyperplasia do not appear to result from differences in the levels of circulating E2, progesterone, or PRL in the E2-treated ACI and COP rats.

Mammary Cancers Induced in ACI Rats by E2 Exhibit Genomic Instability. Flow cytometric analysis of cellular DNA content within mammary cancers induced in ACI rats by prolonged treatment with E2 indicated that the majority (67%) of these tumors exhibited aneuploidy (Fig. 4). Of 15 mammary cancers analyzed, 5 (33%) exhibited a normal DNA profile where the great majority of cells displayed a diploid DNA content (Fig. 4A); 7 (47%) cancers contained a significant fraction of cells that exhibited a hyperdiploid DNA content (Fig. 4B); 2 (13%) contained a large fraction of cells with tetraploid DNA content (Fig. 4C); and 1 (7%) contained a large fraction of cells that were hypertetraploid in their DNA content (Fig. 4D).

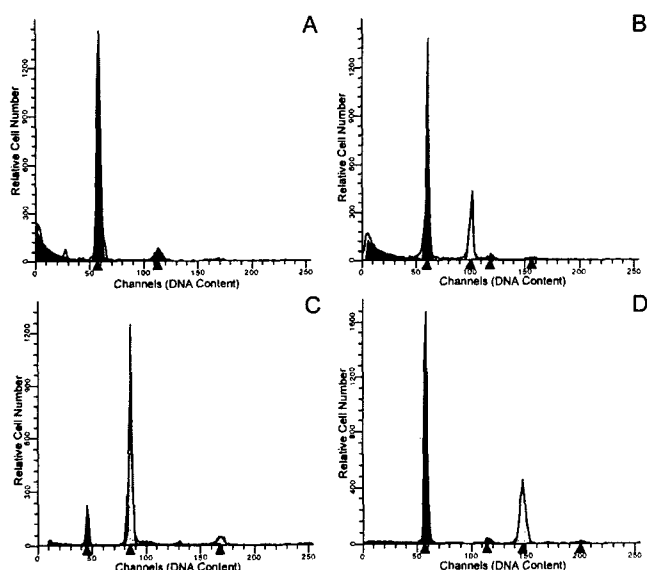


Fig. 4. Mammary cancers induced in ACI rats by E2 exhibited aneuploidy. Mammary cancers were induced in ovary-intact ACI rats by prolonged treatment with E2. The horizontal axis represents an arbitrary scale of relative fluorescence intensity, which is directly proportional to DNA content. The vertical axis indicates relative cell number. Cells from the spleen of each tumor-bearing animal were included in parallel samples to provide internal standards for diploid and tetraploid DNA content. (A) Mammary cancer from an ACI rat treated with E2 for 158 days. The majority of cells ($\approx 96\%$) were diploid. (B) Mammary cancer from an ACI rat treated for 102 days. Approximately 68% of cells were diploid, whereas $\approx 32\%$ of cells were aneuploid with hyperdiploid DNA content. (C) Mammary cancer from an ACI rat treated for 202 days. Only $\approx 12\%$ of cells were diploid, whereas $\approx 88\%$ exhibited a tetraploid DNA content. (D) Mammary cancer from an ACI rat treated for 268 days. The majority of cells ($\approx 65\%$) were diploid, whereas $\approx 35\%$ were aneuploid with a hypertetraploid DNA content.

Estrogen-Induced Mammary Cancers in ACI Rats Are Dependent on Exogenous E2. To determine whether continuous exposure to exogenous E2 is required for maintenance of established mammary cancers, the E2-containing implants were removed from a group of 4 tumor-bearing ACI rats harboring a total of 14 palpable mammary tumors. All of the mammary cancers regressed following implant removal. The regression of four tumors in one animal is illustrated in Fig. 5. In that animal, tumors 1, 2, 3, and 4 were detected following 98, 133, 147, and 154 days of E2 treatment, respectively. The E2-containing implant was removed on day 158 relative to initiation of estrogen treatment, when the tumors ranged from 0.4 to 1.1 cm in diameter. Within 38 days of implant removal, the largest of the four tumors had regressed to 0.2 cm in diameter and the three smaller tumors were no longer detectable by palpation. Regression of the largest tumor was complete within 59 days after removal of the E2-containing implant. These data, which are representative of those from three additional tumor-bearing animals, indicate that maintenance of elevated levels of circulating E2 are required for continued growth of E2-induced mammary cancers.

Estrogen-Induced Atypical Epithelial Hyperplasia and Mammary Cancers in ACI Rats Express PR. The mammary glands of ACI rats treated with E2 for as few as 12 wk contained focal regions of atypical epithelial hyperplasia in which the cells exhibited enlarged nuclear and cellular volumes and dense eosinophilic cytoplasmic staining (Fig. 6A). In contrast, no such foci were observed in the mammary glands of COP rats that were treated with E2 for 12 wk (data not shown). The majority of epithelial

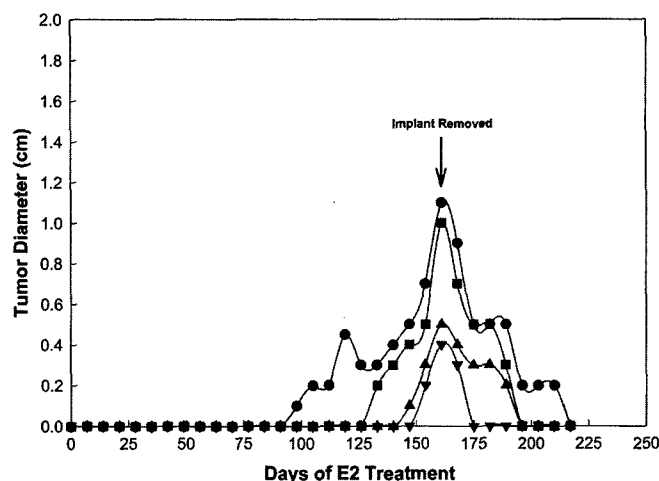


Fig. 5. Mammary cancers induced in ACI rats by E2 regressed upon removal of the hormone-containing implant. The four different symbols illustrate the development of four E2-induced mammary cancers in an individual rat and their subsequent regression following removal of the E2-containing implant 158 days after initiation of E2 treatment. Three of the tumors (■, ▲, ▼) regressed completely within 38 days of implant removal, whereas the largest tumor (●) regressed completely within 59 days of cessation of E2 treatment.

cells within the atypical foci observed in E2-treated ACI rats displayed strong immunoreactivity to an antibody to the PR (Fig. 6B). Similarly, the majority of epithelial cells within the mammary cancers that developed in response to prolonged E2 treatment also stained intensely for the presence of PR, regardless of whether they were of the comedo (Fig. 6C), papillary (Fig. 6D), or cribriform (data not shown) types. By contrast, only 20–30% of the epithelial cells within the hyperplastic lobules of ACI rats treated for 12 wk (Fig. 6E) or longer (Fig. 6D) with E2, or normal ductal epithelial cells of age-matched untreated controls (Fig. 6F) stained positive for PR. These data suggest that the atypical foci observed within 12 wk of initiation of E2 treatment may be precursors of the E2-induced carcinomas. The number of focal regions of atypical epithelial hyperplasia and the degree of cellular atypia within the foci were observed to increase in the mammary glands of female ACI rats as a function of the length of E2 treatment beyond the 12-wk time point. Consequently, atypical hyperplastic foci were numerous in ACI rats bearing E2-induced mammary cancers (data not shown).

Discussion

The genetically related ACI and COP rat strains exhibit diametrically opposed phenotypes with respect to susceptibility to E2-induced mammary cancers (15, 16). In the present study, we have demonstrated that the mammary gland of the highly susceptible ACI rat exhibits a greater proliferative response to administered E2 compared with the resistant COP rat. This difference was evidenced by quantification of the number of mammary cells incorporating BrdUrd as well as by histologic indicators of lobuloalveolar hyperplasia. The number of BrdUrd-positive cells was 30% greater in E2-treated ACI rats than in E2-treated COP rats; the difference (fold induction) between basal and E2-stimulated mammary cell proliferation in ACI rats was more than twice that observed in the COP strain; and the percentage of the mammary gland cross-sectional area comprised of parenchyma was 30% greater in E2-treated ACI rats than in treated COP rats. Moreover, focal regions of atypical epithelial hyperplasia were observed in the mammary glands of ACI, but not COP, rats that were treated with E2 for 12 wk. The associations between estrogens, cell proliferation, and breast cancer risk in human populations and mammary cancer devel-

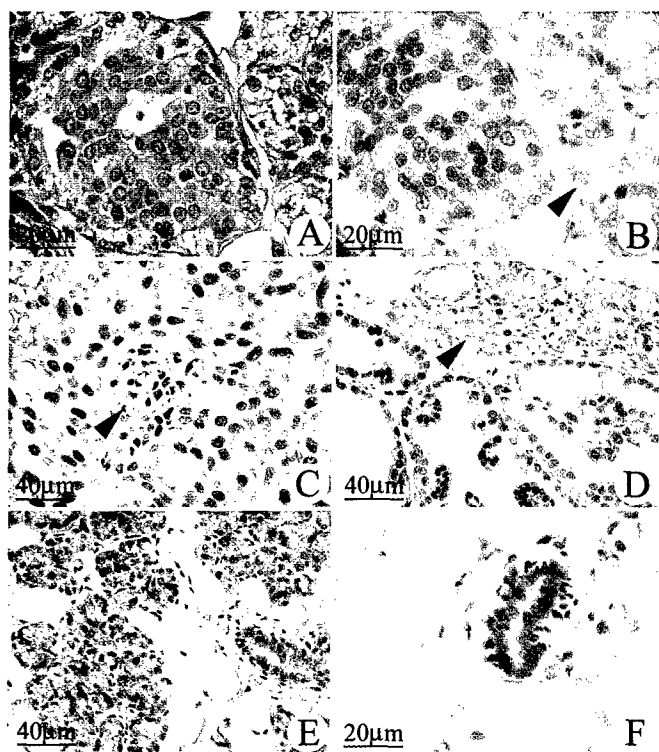


Fig. 6. Atypical epithelial hyperplasias and mammary cancers induced in ACI rats by E2 express PR. Cells expressing PR were identified immunohistochemically as described in *Materials and Methods*. (A) Mammary gland, sectioned and stained with hematoxylin and eosin, from a 21-wk-old female ACI rat treated with E2 for 12 wk. Illustrated is a focal region of atypical epithelial hyperplasia and, to the right, adjacent acinar structures. The epithelial cells within the atypical hyperplasia exhibited enlarged nuclei and dense eosinophilic cytoplasmic staining. The illustrated atypical hyperplasia was minimally deviated relative to the surrounding lobules. The number of atypical hyperplastic foci and the degree of cellular atypia were observed to increase as a function of the duration of E2 treatment beyond 12 wk (data not illustrated). (B) A serial section to that in A that has been immunostained for PR. The majority of cells within the atypical epithelial hyperplasia exhibited immunoreactivity to PR, whereas fewer cells in the adjacent acinar structures stained positive for PR (arrow). (C) Mammary comedo carcinoma from an ACI rat treated with E2 for 193 days. The majority of the cancer cells exhibited immunoreactivity to PR. The arrow indicates necrotic debris characteristic of these cancers. (D) Mammary papillary carcinoma from an ACI rat treated with E2 for 216 days. The majority of the epithelial cells within the cancer exhibited immunoreactivity to PR. The arrow indicates adjacent acinar structures where a subset of the epithelial cells were immunoreactive to PR. (E) Mammary gland from an ACI rat treated with E2 for 12 wk exhibited lobuloalveolar hyperplasia. A subset of the epithelial cells stained positive for PR. (F) Mammary gland from an untreated, 21-wk-old, ovary-intact ACI rat; age-matched control for the E2-treated animals illustrated in A, B, and E. A subset of epithelial cells within the normal ductal structures exhibited immunoreactivity to PR.

opment in experimental models have long been recognized (24–28). However, the data presented herein illustrate a genetically conferred difference in the ability of E2 to induce cell proliferation, lobuloalveolar hyperplasia, and atypical epithelial hyperplasia within the mammary glands of two genetically related inbred rat strains that differ in their susceptibility to E2-induced mammary cancers. Although the observed difference in the responsiveness of the ACI and COP mammary glands to administered E2 correlates with the differing susceptibilities of these strains to E2-induced mammary cancers, a cause and effect relationship cannot be concluded from the available data.

The quantitative and qualitative differences in the response of the mammary tissues of the ACI and COP rat strains to E2 did not appear to result from differences in circulating E2, proges-

terone, or PRL. Hyperprolactinemia has been shown to be sufficient to induce mammary cancer in certain strains of mouse (29–31) and rat (32, 33). Because rats treated chronically with estrogens to induce mammary cancers often develop PRL-producing pituitary tumors, it has been hypothesized or assumed that pituitary tumor-associated hyperprolactinemia is the proximal causative hormonal factor in estrogen-induced mammary carcinogenesis (12, 34–37). Data presented herein and published previously (16) illustrate that E2 induces pituitary tumors and associated hyperprolactinemia in female COP rats but does not induce a high incidence of mammary cancers. Moreover, ovariectomy (15) and dietary energy restriction (38) each inhibit development of E2-induced mammary cancers in female ACI rats without inhibiting development of pituitary tumors and associated hyperprolactinemia. Therefore, hyperprolactinemia is insufficient to induce a high incidence of mammary cancers in estrogen-treated rats. It is interesting to note that the genetic loci that confer and/or modulate susceptibility to E2-induced mammary cancers in F₂ progeny from crosses between the highly susceptible ACI strain and two resistant rat strains, COP and Brown Norway, appear to be distinct from those that influence development of estrogen-induced pituitary tumors, indicating that these two estrogen-induced tumorigenic processes may be genetically separable (unpublished data).

In the present study, female ACI and COP rats exhibited similar increases in pituitary weight and circulating PRL in response to 12 wk of E2 treatment. By contrast, male ACI rats exhibit a significantly greater pituitary growth response to diethylstilbestrol (DES) than do male COP rats (39). The ACI rat appears not to display a significant sexual dimorphism in relation to the pituitary growth response to administered estrogen (our unpublished data). Consequently, the amount of E2-induced pituitary growth exhibited by female COP rats closely approached that exhibited by female ACI rats.

The majority of the E2-induced mammary cancers examined in this study exhibited aneuploidy. To our knowledge, this is the first report of genetic instability in mammary cancers induced in any rat strain by administered E2. Preliminary data suggest that loss of heterozygosity (LOH) is a common feature of E2-induced mammary cancers in ACI rats (unpublished data). The roles of aneuploidy and LOH in the development of E2-induced mammary cancers are not understood. Aneuploidy and LOH are commonly observed in human breast cancers (40).

Mammary cancers induced by E2 in ACI rats regressed upon removal of the E2-containing implants, indicating that these cancers are dependent upon exogenous estrogen for their maintenance. Shellabarger *et al.* (41) noted a similar observation for mammary cancers induced in ACI rats by DES, although data were not presented to indicate how rapidly regression occurred. Not established in these studies is whether and to what extent production of estrogens by the ovaries resumed after cessation of prolonged estrogen treatment.

The mammary glands of ACI rats treated with E2 for as few as 12 wk contained focal regions of atypical epithelial hyperplasia. The majority of cells comprising these foci were quite uniform in their expression of PR, relative to cells in the adjacent hyperplastic lobules. The epithelial cells within E2-induced mammary carcinomas were also uniform in expressing PR, suggesting a possible link between the atypical hyperplastic foci and the carcinomas. Atypical epithelial hyperplasia of this type has not been described previously in the mammary glands of estrogen-treated ACI rats. The functional significance of PR and its hormone ligands within the atypical hyperplastic foci and mammary cancers induced in ACI rats is unknown. It is interesting to note that cell proliferation in the human breast is greatest during the luteal phase of the menstrual cycle, when circulating progesterone is highest (42–43). Moreover, ovariectomy markedly inhibits development of mammary cancers in

ACI rats treated with E2 (15) or DES (9). These data suggest that estrogens and progestins may interact in regulating mammary cell proliferation in the human and rat. However, Segaloff (9) demonstrated that treatment of ovariectomized ACI rats with DES and progesterone did not induce mammary cancer development to the extent observed in DES-treated, ovary-intact ACI rats. Because circulating progesterone was not measured by Segaloff (9), it should not be assumed that progesterone levels in the treated ovariectomized rats were restored to within the physiologic range of ovary-intact rats.

In summary, the mammary epithelia of ACI and COP rats exhibited quantitative and qualitative differences in their responsiveness to E2. These differences correlated with the differing susceptibilities of these two rat strains to E2-induced mammary cancers. Because the ACI rat appears unique in its high degree of susceptibility to E2-induced mammary cancers,

whereas the genetically related COP rat is resistant to these cancers, these two rat strains provide useful models for studying the role of E2 in mammary cancer development. Ongoing genetic studies are addressing the mechanism underlying these strain differences in the belief that this information will prove relevant to our understanding of the etiology of human breast cancers.

We thank Dr. Karen Gould for her valuable comments during the preparation of this manuscript, Dr. Charles Kuszynski for assistance with flow cytometry, and David Heard, John Schoeman, Dondi Holland, and Connie Thomas for their assistance in animal care. This research was supported by Grants R01CA68529, R01CA77876, and P30CA36727 from the National Institutes of Health; DAMD17-98-1-8217 from the U.S. Army Breast Cancer Research Program; and 97A-146 from the American Institute for Cancer Research. D.M.E.H. and T.E.S. were supported by National Institutes of Health Grant T32CA09476.

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ALLELIC IMBALANCE IN MAMMARY TUMORS INDUCED IN ACI/COPENHAGEN F1 RATS BY 17 β -ESTRADIOL. Lisa A. Flood, Karen L. Pennington and James D. Shull. *University of Nebraska Medical Center, Omaha, NE 68198-6805*

The ACI and Copenhagen (COP) rat strains exhibit opposing susceptibilities to mammary cancers induced by continuous treatment with 17 β -estradiol (E2). We have recently demonstrated that the unique susceptibility of the ACI rat to E2-induced mammary cancers behaves as an incompletely dominant trait in reciprocal crosses to the COP strain. Although virtually all of the female ACI/COP F1 progeny develop mammary cancers in response to E2, latency is delayed and tumor burden is reduced in F1 females relative to that observed in ACI females. Linkage analysis reveals on rat chromosome 5 a locus, *Emca1* (*E*strogen-induced *m*ammary *c*ancer1), that modifies susceptibility to E2-induced mammary cancers in ACI x COP intercrosses. The mammary tumors that develop in E2 treated rats are estrogen-dependent and frequently exhibit aneuploidy. We hypothesize that deletion of COP alleles within *Emca1* and/or elsewhere in the genome may contribute to E2-induced mammary carcinogenesis in ACI/COP F1 females. A panel of 21 mammary tumors induced in F1 females by E2 was examined for allelic imbalance (AI) at polymorphic loci distributed across the rat genome. DNA from each tumor was amplified using PCR and primer pairs flanking mapped microsatellite repeats, the radiolabeled DNA products were separated on acrylamide gels, and the amount of products arising from the ACI and COP alleles were quantified on a phosphorimager and compared to results generated from DNA prepared from the spleen. AI, defined as an ACI/COP allele ratio in a tumor that differs from that of the spleen by $\geq 25\%$, was evaluated for each chromosome, and representative data are summarized here. For each of 17 markers evaluated on rat chromosome 5, AI was observed in at least 1 and as many as 7 of the 21 tumors. On average, an individual tumor exhibited AI at 20% (range 0-82%) of the markers. Approximately 65% of the observed AIs resulted from loss of COP alleles (or gain of ACI alleles). Only 1 tumor did not exhibit any AI, 2 tumors exhibited AI at approximately 50% of the markers and 2 tumors exhibited AI at a majority of the chromosome 5 markers. Within the 4 tumors exhibiting AI at 50% or more of the markers, 3 tumors exhibited consistent loss of COP alleles and 1 exhibited consistent loss of ACI alleles. AI was also observed in 14-55% of the 21 tumors at each of 6 markers on chromosome 18. Only 2 tumors did not exhibit AI at at least 1 marker, whereas 5 tumors exhibited AI at 50% or more of the chromosome 18 markers. Approximately 75% of the AI events on chromosome 18 resulted from loss of COP alleles. We conclude that widespread genomic instability is a common feature of E2-induced mammary cancers. Supported by grants DAMD17-98-1-8217, R01-CA68529 and R01-CA77876.



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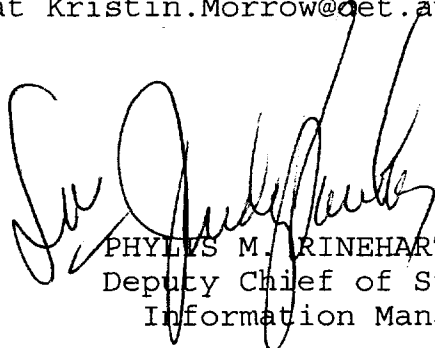
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